

GENETIC AND MOLECULAR ANALYSIS OF  
FERTILITY RESTORATION AND INSTABILITY IN  
CYTOPLASMIC MALE STERILE Phaseolus vulgaris L.

By

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GENETIC AND MOLECULAR ANALYSIS OF FERTILITY RESTORATION  
AND INSTABILITY IN CYTOPLASMIC MALE STERILE  
Phaseolus vulgaris L.

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Cytoplasmic male sterility (CMS) is valuable both in hybrid seed development and in studies of cytoplasmic-nuclear interaction. The potential of CMS in Phaseolus vulgaris was investigated with both objectives in mind. The cytoplasmic male sterile phenotype in Phaseolus vulgaris was relatively stable when backcrossed to 'Sprite' snap bean maintainer nuclear background (CMS-Sprite). However, spontaneous cytoplasmic reversion to fertility was observed and characterized.

A simply inherited restorer was identified in line R-351. Only partial restoration of fertility (semisterility) was achieved in the  $F_1$  generation (CMS-Sprite x R-351). Fully fertile restored plants were observed in the  $F_2$  population in a ratio of 3:1 (fertile:sterile).

The fertile plants produced in the  $F_2$  population appeared to be irreversibly restored: Segregation for sterility was not observed in testcross ( $F_2$  x maintainer) or selfed populations from  $F_2$  plants heterozygous for restorer. This suggests that a cytoplasmic alteration occurs in the process of restoration by a nuclear gene.

Restriction endonuclease analysis of mitochondrial DNA from two independent fertile revertants revealed the loss of a 6.5 kilobase PstI/SstII fragment indicating mitochondrial genome reorganization. Examination of one fertile restored population showed the loss of the same fragment. This suggests that the molecular basis of both phenomena, reversion and restoration, may be associated with mitochondrial genome rearrangement.

## CHAPTER I INTRODUCTION

Cytoplasmic male sterility (CMS) can be described as premature pollen abortion resulting from a cytoplasmic-nuclear incompatibility. Since the alteration is cytoplasmic segregation for fertility does not occur in progeny of a cross between the CMS source and a fertile pollen donor of maintainer genotype. Sources of CMS have been developed in a number of species and have been useful in hybrid seed development.

More recently, CMS has become important in providing information about the cytoplasm. Nuclear genes that are able to override the CMS phenotype and restore male fertility exist in nature. Fertility restoration by a nuclear gene over a cytoplasmic mutation provides an exceptional system for analysis of interactions between nucleus and cytoplasm.

The purpose of this research was to provide a better understanding of the genetic behavior of CMS in Phaseolus vulgaris. This includes study of the genetics of restoration and instability of the CMS phenotype. The possibilities of viral involvement in CMS or detection of cytoplasmic alteration at a molecular level were also investigated.

## CHAPTER II LITERATURE REVIEW

### Cytoplasmic Inheritance In Higher Plants

Cytoplasmic markers have provided a means of studying patterns of cytoplasmic inheritance in higher plants, a step in understanding the role of cytoplasmic genes in plant development and function. Cytoplasmic markers available include tentoxin sensitivity (Durbin and Uchytıl, 1977), triazine resistance (Pfister et al., 1981), and streptomycin resistance (Maliga et al., 1973), all of which are encoded within the chloroplast genome. Only a few selectable mitochondrial markers have yet been identified.

In the majority of all genera organelles appear to be maternally inherited (Kirk and Tilney-Bassett, 1978) such that cytoplasmic mutations are transmitted only through the female in a cross. However, a number of species demonstrating biparental plastid inheritance do exist. These include Pelargonium spp., Oenothera spp., Rhododendron spp., and Secale cereale (Gillham, 1978). Plastid inheritance has been reported to be biparental in Phaseolus

vulgaris (Parker, 1933). The degree of biparental inheritance is thought to be related to the number of organelles included within the generative cell of the male gametophyte (Hagemann, 1976). Another theory, originally developed from studies of Chlamydomonas, suggests that uniparental inheritance is achieved by selective destruction of organelles from one parent (Sager and Kitchin, 1975; Sears, 1980; Vaughn et al., 1980; Day and Ellis, 1984).

Most studies of cytoplasmic inheritance have dealt with plastid markers due to the lack of easily detectable mitochondrial mutations. In a number of species cytoplasmic male sterility (CMS) has been identified as a probable mitochondrial lesion (Levings and Pring, 1976; Warmke and Lee, 1977; Clark et al., 1985; Galun et al., 1982; Leaver and Gray, 1982; Hanson and Conde, 1985). Thus, CMS provides an experimentally feasible approach to studying inheritance of the mitochondrial genome.

#### Characteristics of Cytoplasmic Male Sterility

Cytoplasmic male sterility occurs in more than 150 species (Edwardson, 1956; Edwardson, 1970; Laser and Lersten, 1972). CMS is the result of a cytoplasmic mutation leading to the inability of an otherwise phenotypically normal plant to produce viable pollen. Although CMS has been reported to arise spontaneously, it usually occurs as a

result of intergeneric, interspecific, or even intra-specific crosses, often followed by recurrent backcrossing (Edwardson, 1970). It is generally believed that such crosses result in a cytoplasmic-nuclear incompatibility manifested in pollen abortion.

Pollen abortion in CMS species does not always take the same form. Laser and Lersten (1972) reviewed the different stages in pollen development at which abortion may take place due to CMS. In some cases the tapetum appears to be the first site of malfunction. In CMS Sorghum (Brooks et al., 1966; Overman and Warmke, 1972) and Triticum (Heyne and Livers, 1968) premature tapetal breakdown was directly associated with pollen abortion. In CMS-T maize tapetal mitochondria appear to enlarge and degenerate prematurely when compared to tapetal mitochondria of male fertile maize (Warmke and Lee, 1977). Colhoun and Steer (1981) suggested that the entire course of tapetal cell development has been modified, perhaps by hormonal changes. The tapetum is thought to be a source of nutrients for developing microspores. Environmental factors inducing abnormal tapetal development are usually followed by pollen abortion, demonstrating the crucial relationship between normal tapetal function and pollen development (Vasil, 1967).

In CMS-T maize accumulation of hydroxycinnamic acid amines (HCA) is inhibited. HCA levels are restored to normal when fertility restorer genes are introduced into the CMS line (Martin-Tanguy et al., 1982). A close correlation between HCA accumulation and maximal rate of RNA, DNA, and protein synthesis is observed in fertile maize; yet whether decreased HCA levels are a cause or result of CMS is not known. In Lycopersicon CMS is associated with structural differences in filament and anther tissues between fertile and CMS plants (Anderson, 1963). Changes in the filaments and vascular bundles of stamens may result in premature microspore degeneration.

Nicotiana provides a well-documented example of morphological changes directly associated with CMS. In CMS Nicotiana anthers may become "feminized" and extend into nonfunctional stigmas or even ovules (Chaplin, 1964; Clayton, 1950). Anthers may also appear petaloid. CMS in Nicotiana is generally alloplasmic (resulting from interspecific hybridization). In some Nicotiana CMS lines, developed by displacing the nuclear background of one species (eg. N. tabacum) with that of another (eg. N. glutinosa), recurrent backcrossing to the original nuclear background (N. tabacum) may not restore fertility or the original floral morphology (Burk, 1960). This inability of the original nucleus to restore fertility to its native

cytoplasm is indication of a cytoplasmic alteration induced by the process of introducing a foreign nuclear genotype.

In petunia a possible cause of pollen abortion was proposed by Izhar and Frankel (1971). Activation of callase for proper dissolution of callose requires a decrease in pH within the anther locule. Faulty timing of such a pH change may result in either premature or delayed callose dissolution, causing abortion in tetrads.

Virus infection has been considered a cause of pollen abortion associated with CMS in some species. Severity of virus disease symptoms is variable; evidence exists to suggest CMS may be the result of virus infection causing pollen abortion without other observable viral symptoms (Atanasoff, 1971).

#### Uses of Cytoplasmic Male Sterility

The primary practical value of cytoplasmic male sterility has been in hybrid seed production. Since CMS plants are unable to self-pollinate, the plant breeder can be assured that seed collected from a CMS line is the result of cross-pollination, eliminating the labor of hand emasculation. A hybrid seed program using CMS requires a stable source of CMS; a "maintainer" genotype which, when crossed to the CMS line, produces 100% male-sterile  $F_1$

progeny; and a "restorer" genotype which contains nuclear genes able to countermand the cytoplasmic mutation and produce 100% fertile  $F_1$  progeny. Such hybrid seed programs have been developed in onion, sugar beet, maize, carrot, sorghum, and sunflower.

CMS has provided a valuable mutation for the study of cytoplasmic-nuclear interaction. In Nicotiana, CMS results from interspecific hybridization of N. repanda x N. tabacum followed by recurrent backcrossing with N. tabacum as pollen donor. A fragment of a single N. repanda chromosome is required to restore fertility. The nucleolar organizer region (NOR) of the N. repanda chromosome appears to be the critical region involved in restoration (Gerstel et al., 1978). The association of NOR with restoration has also been observed in other Nicotiana CMS combinations (Burns and Gerstel, 1981). These observations suggest that incompatibility between nucleolar organizer chromosomes and organelles may to some extent be responsible for CMS.

An interesting aspect of CMS with respect to nuclear-cytoplasmic interaction was observed in maize by Rhoades (1943). Rhoades first described a cytoplasmic mutation which was induced by a nuclear mutator gene iojap and produced a male-sterile phenotype. CMS has since been observed in a cross between a fertile inbred R181 and a stock carrying the recessive nuclear gene iojap (Lemke et al., 1985).

### Restoration of Fertility

Restoration of fertility in a CMS line is conditioned by a nuclear genotype able to compensate for the incompatibility between nucleus and cytoplasm. In general, restorer genes can be used to differentiate cytoplasms. CMS maize can be divided into three main cytoplasm types: CMS-T, restored by  $\underline{Rf}_1\underline{Rf}_2$ ; CMS-S, restored by  $\underline{Rf}_3$ ; and CMS-C restored by  $\underline{Rf}_4$  with perhaps another factor (Duvick, 1965; Beckett, 1971). Restoration in CMS-T is sporophytic, or conditioned by a product of the maternal tissue. That is not the case in CMS-S where restoration by  $\underline{Rf}_3$  is gametophytic; pollen viability depends on the genotype of the individual microspore and  $\underline{Rf}_3\underline{rf}_3$  plants produce only 50% viable pollen (Duvick, 1965; Buchert, 1961).  $\underline{Rf}_1, \underline{Rf}_2$ , and  $\underline{Rf}_3$  have been mapped to chromosomes 3 (Duvick et al., 1961), 9 (Snyder and Duvick, 1969), and 2 (Laughnan and Gabay, 1978), respectively.

In petunia, two restorer systems exist for a single cytoplasm. The first is polygenic, demonstrating complementary gene action and temperature sensitivity (Van Marrewijk, 1969; Izhar, 1977). In this restorer system the number and combination of restorer genes is influential on the stage of microspore breakdown (Izhar, 1977). The second restorer involves a single dominant gene (Edwardson and Warmke, 1967). This single gene restoration is neither

temperature-sensitive nor dependent on genetic background (Izhar, 1978).

In Vicia faba, two sources of CMS have been identified: 350 (Berthelem, 1970, as cited in Thiellement, 1982) and 447 (Bond et al., 1966). The expression of CMS in line 447 has been correlated with the presence of virus-like cytoplasmic RNA molecules which will be described later. Restoration by a single dominant restorer gene results in irreversible full fertility and disappearance of the RNAs (Bond et al., 1966; Edwardson et al., 1976). In  $F_2$  populations all progeny are 100% fertile with no segregation for the expected 25% male-sterile plants; a permanent cytoplasmic reversion to fertility occurs upon introduction of the nuclear restorer. Line 447 also demonstrates a high level of instability and spontaneous reversion to fertility and semi-sterility (Thiellement, 1977).

Biochemical analysis of restoration in CMS barley has raised another possibility for the means of restoration. Two CMS sources exist in barley: msm1 and msm2. Restoration in barley cytoplasm msm1 is conditioned by a dominant Rfmla gene (Ahokas, 1980). Rfmla restoration appears to correlate with an increase in bound cytokinin levels in root exudates of plants at anthesis. CMS plants have significantly lower levels of the cytokinins than normal fertile plants; this

suggests a possible role of phytohormones in expression of CMS (Ahokas, 1982).

### Spontaneous Reversion

Spontaneous reversion of CMS plants to fertility has been described in a number of species including maize CMS-S (Laughnan and Gabay, 1973), pearl millet (Burton, 1972; 1977), sunflower (LeClercq, 1971; 1979), and faba bean (Bond et al., 1966; Thiellement, 1980). In most cases reversion is cytoplasmic, with no apparent nuclear mutation. Exceptions are maize CMS-S and pearl millet.

In maize CMS-S, spontaneous reversion to fertility may be a cytoplasmic or a nuclear event (Laughnan and Gabay, 1973). CMS-S is unique among the maize sterile cytoplasms in that mitochondrial DNA preparations reveal the presence of two linear double-stranded plasmid-like DNA molecules of 6397 bp and 5453 bp (Pring et al., 1977; Paillard et al., 1985; Levings and Sederoff, 1983). These molecules have been designated S1 and S2 respectively. These S1 and S2 molecules are no longer apparent as free molecules in mitochondrial DNA preparations of cytoplasmic revertants (Levings et al., 1980).

Spontaneous reversion may also be nuclear in CMS-S maize (Laughnan and Gabay, 1973). In fact, nuclear reversion events, or mutations resulting in restorer gene activity, have been mapped to a number of nuclear sites other than the

chromosome 2 site of Rf<sub>3</sub> (Laughnan and Gabay, 1975). The rate of reversion, both nuclear and cytoplasmic, is under nuclear control; reversion frequencies, as well as the proportion of reversion events that are nuclear, vary depending on nuclear background (Laughnan et al., 1981).

Nuclear and cytoplasmic reversions have also been reported in pearl millet (Burton, 1972), with nuclear background strongly influencing reversion frequency (Clement, 1975). Such reversion events are valuable in the study of CMS in part because they provide a fertile plant line which is theoretically identical to the CMS line except at the point of mutation.

In Vicia faba, cytoplasmic instability produces fully fertile as well as semi-sterile plants (Bond et al., 1966; Thiellement, 1982). This instability, as well as CMS itself, appears to be correlated with the presence of virus-like cytoplasmic inclusions that are no longer present upon reversion (Edwardson et al., 1976).

In CMS-T maize the "reversion" event, or mutation to fertility, is not spontaneous but induced by organogenic callus culture. Maize CMS-T is the most extensively studied CMS system. Discovered in 1944 in an inbred variety "Golden June" (Duvick, 1965) and used for many years in hybrid corn production, CMS-T was found to be susceptible to the southern corn leaf blight pathogen Helminthosporium

maydis race T. The importance of this susceptibility, aside from its economic impact, is that it is found in extremely tight linkage, perhaps even pleiotropic, to the CMS lesion. Male-fertile plants can be regenerated from CMS-T callus, with or without selection pressure; these fertile mutants are also resistant to the pathotoxin produced by H. maydis (Gengenbach et al., 1977; Pring et al., 1981; Umbeck and Gengenbach, 1983). These mutations are cytoplasmic and have not been observed to occur spontaneously.

The observation of reversion events in such a number of CMS systems raises questions about the structure of the mitochondrial genome. In higher plants, the mitochondrial genomes are larger and more variable in size than in other eukaryotes (Ward et al., 1981; Levings, 1983). The plant mitochondrial genome undergoes intra- and inter-molecular recombinations between repeated sequences resulting in a number of different sized molecules within the mitochondrion (Lonsdale, 1984). Such recombination events may be involved in spontaneous cytoplasmic reversion.

### Molecular Analysis of CMS

Analysis of cytoplasmic male sterility has been approached using physiological, morphological, and biochemical techniques in order to determine an underlying mechanism. Although these approaches have yielded valuable information about abnormalities involved in the CMS phenotype, most have been observations secondary to the actual mutational event. Analysis of CMS at a molecular level has been most valuable in approaching the actual initial event responsible for CMS.

The earliest theories dealing with the cause of CMS focused on viral involvement (reviewed by Atanasoff, 1971). Although graft transmissibility of CMS has been reported in a number of species, studies of CMS Vicia faba have provided the most convincing evidence of virus infection. In Vicia faba CMS line 447, "cytoplasmic spherical bodies" (CSB) about 70 nm in diameter have been observed by electron microscopy in cytoplasm preparations of CMS material. The CSBs are not observed in normal fertile, restored, or revertant tissue (Edwardson et al., 1976). The CSBs were later found to contain double-stranded RNA molecules of approximately  $10-13 \times 10^6$  MW (Grill and Garger, 1981; Scalla et al., 1981). <sup>35</sup>(S)Methionine in organello

labelling of mitochondrial proteins reveals a unique polypeptide present in CMS lines only (Boutry and Briquet, 1982). This protein may be the product of the virus-like elements or of the CMS V. faba mitochondrial genome itself, since restriction endonuclease digests of CMS V. faba mitochondrial DNA differ from those of fertile V. faba by a number of fragments.

Restriction endonuclease analysis has been useful for identification of the site involved in CMS. In CMS-T maize numerous restriction pattern differences exist between CMS and normal mitochondrial DNA, while chloroplast DNA restriction patterns are nearly identical, suggesting mitochondrial involvement in CMS (Levings and Pring, 1976). Comparisons of mitochondrial DNA patterns between CMS-T and fertile mutants regenerated from tissue culture reveal a 6.7 kilobase XhoI fragment absent from 15 of 16 fertile mutants but present in CMS-T plants (Umbeck and Gengenbach, 1983). Although evidence of involvement of the 6.7 kilobase fragment in CMS and T-toxin sensitivity is not conclusive, a transcript in this region, unique to CMS-T and deleted or rearranged in fertile mutants, has been identified (Wise et al., 1986). Transcripts in this region also appear to be altered upon restoration (Dewey et al., 1986).

In maize CMS-S, CMS appears to be associated with presence of S1 and S2 linear plasmid-like DNA molecules within the mitochondrion (Pring et al., 1977). Although S1 and S2 appear in higher copy number than the mitochondrial genome (Pring et al., 1977), their replication rates are altered by nuclear background (Laughnan et al., 1981).

S1 and S2 are characterized by identical 208 base pair terminal inverted repeats and a homologous 1.4 kilobase sequence at one end of each molecule (Lonsdale et al., 1981; Levings and Sederoff, 1983; Paillard et al., 1985). Sequences identical to the terminal repeats exist within the mitochondrial genome. This sequence homology allows for recombination with S1 and S2, linearizing the mitochondrial genome (Schardl et al., 1984; Schardl et al., 1985a, 1985b). Transcripts homologous to S2 have been detected (Schardl et al., 1985b). Evidence of S1 and S2 involvement in CMS expression is not conclusive, however. Specific maintainer nuclear backgrounds in which CMS-S cytoplasmic reversion does not involve the loss of free S1 and S2 molecules have been identified. The pattern of mitochondrial genome rearrangement is distinct from that observed in S1 and S2 transposition (Escote et al., 1985).

In CMS sorghum line IS1112C, two mitochondrial plasmid-like linear double-stranded DNA molecules of comparable sizes to S1 and S2 have been designated N1 (5700 bp) and N2 (5300 bp) (Dixon and Leaver, 1982; Pring et al., 1982). Spontaneous reversion to fertility has not been observed in CMS sorghum, however, and a direct relationship of N1 and N2 to CMS has not yet been established (Chase and Pring, 1986).

Another useful technique for analysis of CMS in amenable species has been protoplast fusion. Organelle genome characteristics such as restriction endonuclease patterns can be used to identify organelle components of regenerated somatic hybrids. Protoplast fusion experiments between CMS and fertile lines of petunia with distinct chloroplast DNA restriction patterns have provided conclusive evidence that the CMS mutation resides on the mitochondrial genome; the CMS trait and the chloroplasts characteristic of the CMS line segregate independently in regenerated somatic hybrids (Clark et al., 1985). In other words, fertile regenerated somatic hybrids were identified with chloroplasts characteristic of the CMS line. Independent segregation of CMS and the chloroplast genome has also been observed in CMS Nicotiana (Galun et al., 1982). Fusion experiments have led to identification

of sequences derived from the petunia CMS mitochondrial genome and unique to male-sterile regenerated somatic hybrids (Boeshore et al., 1985). In petunia fusion experiments have allowed for the delineation of a mitochondrial region associated with CMS.

Protoplast fusion experiments have provided evidence of intergenomic mitochondrial (Rothenberg et al., 1985) and chloroplast (Medgyesy et al., 1985) recombination. Although such recombinational events have been proposed to explain homologies between chloroplast and mitochondrial DNA in many species (Stern and Palmer, 1984), fusion experiments provide a method for inducing such intergenomic events.

An important question in cytoplasmic-nuclear interaction is whether heterogeneous organelle populations can exist within a single plant. For example, the question arises in the case of spontaneous cytoplasmic reversion, where the initial mutation for fertility is assumed to occur in a single organelle. At some stage, functional and dysfunctional organelles may coexist within a single revertant. The same question arises in cases of "semi-sterility", or plants intermediate between cytoplasmic male sterile and fertile, such as in CMS Vicia faba (Thielllement, 1982) and petunia (Izhar, 1977; 1978; Van Marrewijk, 1969). In many fusion events apparently random sorting of

chloroplasts to homogeneity takes place, resulting in a somatic hybrid with a single parental chloroplast type (Scowcroft and Larkin, 1981; Aviv et al., 1980; Gleba, 1978), or chloroplast segregation is unidirectional for one parental plastid type (Maliga et al., 1980). However, Izhar et al. (1983) were able to identify heteroplasmic cybrids resulting from protoplast fusions of CMS and fertile Petunia. These heteroplasmic plants were identified by subsequent sorting out in later generations of selfing or from cuttings. The phenotypic expression of heteroplasmic plants is sterility, although the sterility and fertility elements do not exhibit simple dominance-recessive expression in a heteroplasmic state.

Initial interest in the CMS phenotype developed from increasing demand for hybrid seed varieties. Yet CMS has offered a useful system for studying the intricate interaction required between the plant cell nucleus and organelles for cell function. Although the phenotypic expression of CMS is similar among species, physiological, genetic, and molecular analysis have proven each system unique.

CHAPTER III  
CHARACTERISTICS OF A SECOND SOURCE OF FERTILITY  
RESTORATION IN CYTOPLASMIC MALE STERILE  
Phaseolus vulgaris L.

Introduction

Cytoplasmic male sterility (CMS) in Phaseolus vulgaris was first reported by Bassett and Shuh (1982). The source of CMS was first observed at Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia in a planting of accession line G08063 (Singh et al., 1980). The male sterile phenotype was found to be stably maintained using 'Sprite' snap bean nuclear background and was therefore backcrossed to 'Sprite' at least six generations (CMS-Sprite).

Two characteristics of CMS-Sprite have proven valuable in tests for restoration. One is the fact that pollen aborts at the tetrad stage in CMS material. Aborted tetrads with unevenly thickened walls are produced in large numbers in CMS-Sprite and are readily distinguished from single aborted grains induced by heat stress. Another

characteristic of CMS-Sprite is production of parthenocarpic pods. CMS-Sprite will produce large numbers of seedless pods that provide a valuable indicator of partial sterility (semisterility). In plants which are not fully restored, parthenocarpic and seed pods are produced.

Past efforts to identify a stable source of restoration have met with some success. Although screening of 19 commercial cultivars resulted in little or no fertility in  $F_1$  populations (Bassett and Shuh, 1982), screening of 200 selected plant introductions led to identification of one partial restorer (line 346) (Mackenzie, 1984). Restoration by line 346 is controlled by at least three genes with additive and complementary gene action .

In order to facilitate analysis of CMS in common bean, and for easier manipulation, it is important to identify a source of restoration that is more simply inherited. This study was undertaken to determine the genetics of restoration using a breeding line R-351 as restorer.

#### Materials and Methods

In Florida three plant generations per year can be grown by planting two generations in greenhouses in September and January, and one generation in the field in April. Greenhouse plants were grown in four-liter pots. Field plantings were spaced 10 cm in single row plots and maintained under netting to prevent insect pollinations.

In October, 1983, pollen from line R-351 was used to hand-pollinate open flowers of CMS-Sprite. The following January  $F_1$  seed was grown in the greenhouse and plants were observed for seed set. Seed was harvested from  $F_1$  plants and individual  $F_2$  populations were grown in the field in April.

Within  $F_2$  populations, fertile segregants were selected on the basis of three criteria: pollen stainability (IKI stain), pollen shed, and seed set. Pollen from fertile  $F_2$  segregants was used to hand-pollinate CMS-Sprite. Therefore, the initial backcross population was derived from a cross using selected fertile  $F_2$  plants as pollen donors, some of which were probably homozygous for the restorer gene. Individual BC populations were grown in the greenhouse and evaluated for fertility using the same three criteria. The plants within these initial BC populations with highest fertility were backcrossed to CMS-Sprite ( $BC_2$ ) and were allowed to set seed ( $BC_1F_2$ ). Similarly,  $BC_2$  and  $BC_1F_2$  populations were evaluated for fertility and  $BC_2$  plants with highest fertility were backcrossed to CMS-Sprite ( $BC_3$ ). The backcrossing process was repeated to  $BC_4$  and  $F_2$  populations were also evaluated for fertility segregation.

Unusually high temperatures persisting over the period of bud initiation in the field occurred during evaluation of the  $BC_3$  and  $BC_2F_2$  generations. A second  $BC_3$  population (from remnant seed) was later grown in the greenhouse.

Five selected fertile  $BC_3F_2$  plants were reciprocally crossed to a maintainer genotype. This was done by using 'Sprite' snap bean pollen to pollinate fertile  $BC_3F_2$  plants. Pollen from open flowers of the same 5  $BC_3F_2$  plants was used to pollinate CMS-Sprite. These  $BC_3F_2$  plants were also allowed to self-pollinate for  $BC_3F_3$  seed.

Pollen stainability estimates involved squashing anthers from a mature unopened bud in a drop of IKI. Viability was recorded as the percentage of darkly stained individual grains. "Fertile" plants produced 50-100% stainable pollen; "semisterile" plants produced both stainable pollen (20-50%) and aborted tetrads; and "sterile" plants produced less than 20% stainable pollen with nearly all pollen aborted in tetrads. At least 200 pollen grains per bud were counted.

Pollen shed data were obtained by observing the amount of visible pollen on the stigma of an open flower.

"Fertile" plants produced large amounts of gray pollen completely covering the stigma, "semisterile" plants produced small amounts of pollen barely visible, and "sterile" plants produced no visible pollen on the stigma. At least 3 flowers per plant were observed.

Seed set data were taken by observing the number of pods and seeds per pod produced. "Fertile" plants produced numerous well-filled pods with no evidence of parthenocarpy.

"Semisterile" plants produced at least four seed pods and numerous parthenocarpic pods on a single plant. "Sterile" plants produced all parthenocarpic pods except for occasional plants with 1-2 poorly-filled seed pods.

### Results and Discussion

No distinct semisterile class was observed in  $F_2$  populations using R-351. Since only a small number (less than 10%) of  $F_2$  plants could be considered semisterile, the fertile and semi-sterile classes were pooled so that  $F_2$  populations were classified into only two classes.  $F_2$  populations (Table 1) clearly segregated in a 3:1 (fertile:sterile) ratio characteristic of a single gene with complete dominance.

Backcross populations segregated 1:1 (semisterile:sterile) with no fertile plants evident (Table 2). The appearance of a semisterile class rather than the fertile class predicted from  $F_2$  data may be the result of a single gene with incomplete dominant action. However, with 1:1 (semisterile:sterile) segregation in BC populations the conventional prediction for  $F_2$  segregation would be 1:2:1 (fertile: semisterile:sterile). This was not observed. It should also be noted that environment had a marked effect on

Table 1. Inheritance of fertility restoration on sterile cytoplasm, using R-351 as restorer, in  $F_2$  populations.

| Population  | No. of plants | Segregation for fertility |         | $\chi^2$ (3:1) | P    |
|-------------|---------------|---------------------------|---------|----------------|------|
|             |               | Fertile                   | Sterile |                |      |
| $F_2$       | 57            | 45                        | 12      | 0.47           | 0.49 |
| $BC_1F_2^*$ |               |                           |         |                |      |
| 2-19        | 69            | 53                        | 16      | 0.12           | 0.73 |
| 2-24        | 69            | 56                        | 13      | 1.39           | 0.24 |
| $BC_2F_2$   |               |                           |         |                |      |
| 2-19-6      | 8             | 7                         | 1       |                |      |
| 2-19-29     | 42            | 32                        | 10      |                |      |
| 2-19-36     | 45            | 33                        | 12      |                |      |
| 2-19-44     | 20            | 18                        | 2       |                |      |
| 2-19-48     | 6             | 4                         | 2       |                |      |
|             | 121           | 94                        | 27      | 0.46           | 0.50 |
| $BC_3F_2$   |               |                           |         |                |      |
| 2-24-13-7   | 22            | 18                        | 4       |                |      |
| 2-19-44-20  | 21            | 19                        | 2       |                |      |
| 2-19-44-3   | 10            | 8                         | 2       |                |      |
| 2-19-6-1    | 5             | 3                         | 2       |                |      |
| 2-19-36-48  | 6             | 3                         | 3       |                |      |
| 2-19-36-38  | 5             | 2                         | 3       |                |      |
| 2-19-29-14  | 5             | 2                         | 3       |                |      |
|             | 74            | 55                        | 19      | 0.017          | 0.90 |

\*  $BC_1F_2$  populations were derived from self-pollination of CMS-Sprite x fertile  $F_2$  plants.

Table 2. Inheritance of partial fertility restoration on sterile cytoplasm, using R-351 as restorer, in backcross populations.

| Population                 | No. of Segregation for fertility |                     | $\chi^2$ (1:1) | P         |
|----------------------------|----------------------------------|---------------------|----------------|-----------|
|                            | Plants                           | Semisterile Sterile |                |           |
| BC <sub>2</sub> *          |                                  |                     |                |           |
| 2-19                       | 50                               | 30                  | 20             |           |
| 2-24                       | 44                               | 16                  | 28             |           |
|                            | 94                               | 46                  | 48             | 0.84      |
| BC <sub>3</sub> field      |                                  |                     |                |           |
| 2-19-6                     | 16                               | 11                  | 5              |           |
| 2-19-29                    | 24                               | 5                   | 19             |           |
| 2-19-36                    | 46                               | 16                  | 30             |           |
| 2-19-44                    | 20                               | 12                  | 8              |           |
| 2-24-13                    | 8                                | 5                   | 3              |           |
|                            | 114                              | 49                  | 65             | 2.24 0.13 |
| BC <sub>2</sub> greenhouse |                                  |                     |                |           |
| 2-19-41                    | 40                               | 21                  | 19             | 0.10 0.75 |
| BC <sub>4</sub>            |                                  |                     |                |           |
| 2-19-41-2                  | 24                               | 11                  | 13             |           |
| 2-19-41-16                 | 25                               | 14                  | 11             |           |
|                            | 49                               | 25                  | 24             | 0.02 0.89 |

\*BC<sub>1</sub> populations were derived from CMS-Sprite x fertile F<sub>2</sub> plants and were therefore not true backcross populations.

levels of fertility in backcross populations.  $BC_3$  populations grown in the field during extreme heat produced a larger proportion of sterile plants than did  $BC_3$  plants grown in the greenhouse (Table 2). An increase in the percentage of semisterile plants was observed in  $BC_2F_2$  populations grown under similar field conditions, but the proportion of sterile plants remained 25%.

A simple single-gene complete dominance model is not adequate to explain the observations in backcross and  $F_2$  segregations. A backcross population should be made up of 50% sterile (frfr) and 50% semisterile (Frfr) plants. If this is true,  $F_2$  populations should segregate 1 FrFr:2 Frfr:1 frfr and produce 50% semisterile plants. Thus, it appears that in  $F_2$  populations the restorer heterozygotes express full fertility.

The differential expression in the BC and  $F_2$  plants heterozygous for the restorer may be caused by cytoplasmic-nuclear interaction in the maternal parent. The mitochondrial genome is identified as the probable site of alteration causing CMS in several species (Levings and Pring, 1976; Warmke and Lee, 1977; Galun et al., 1982; Clark et al., 1985; for reviews: Leaver and Gray, 1982; Hanson and Conde, 1985). The nuclear restorer gene product may interact with the mitochondrion to restore normal mitochondrial function at the time of microsporogenesis (Flavell, 1974).

In the case of CMS-Sprite, the backcross population is produced by introducing a restorer gene through the pollen to a nonrestoring egg cell nucleus (fr) in a cytoplasm dysfunctional with respect to microsporogenesis (CMS). The product of this cross would be heterozygous for the restorer but the cytoplasm within the first generation of the cross may only be semi-restored. In other words, a generation of "preconditioning" (interaction between dysfunctional cytoplasm and nuclear restorer) may be required for full fertility. This would then predict that a plant heterozygous for a restorer gene (Frfr) with dysfunctional cytoplasm would appear less fertile (as in semisterile  $F_1$  or BC populations) than would a restorer heterozygote on "preconditioned" (restored) cytoplasm (as in  $F_2$  populations).

To test the hypothesis that cytoplasmic "preconditioning" by the restorer gene is required for full fertility, a reciprocal cross was made. Pollen from five fertile  $BC_3F_2$  plants was used to pollinate CMS-Sprite. 'Sprite' maintainer pollen was then used to pollinate buds of the same five  $BC_3F_2$  plants. Selfed seed from these same 5 plants was also collected.

A 1:1 segregation (semisterile:sterile) was observed in all CMS-Sprite x fertile  $BC_3F_2$  populations (Table 3), suggesting that all 5 selected  $BC_3F_2$  plants were

Table 3. Segregation for partial fertility in  $F_1$  populations produced from testcrosses of CMS-Sprite with 5 selected fertile R-351 derived  $BC_3F_2$  plants.

| Population    | No. of plants | Segregation for fertility |         |  | $\chi^2(1:1)$ | P    |
|---------------|---------------|---------------------------|---------|--|---------------|------|
|               |               | Semisterile               | Sterile |  |               |      |
| CMS-Sprite x  |               |                           |         |  |               |      |
| 2-24-13-7-1   | 14            | 8                         | 6       |  | 0.28          | 0.60 |
| 2-24-13-7-12  | 15            | 7                         | 8       |  | 0.06          | 0.81 |
| 2-24-13-7-14  | 15            | 11                        | 4       |  | 3.26          | 0.07 |
| 2-19-44-20-12 | 14            | 6                         | 8       |  | 0.28          | 0.60 |
| 2-19-44-3-3   | 14            | 7                         | 7       |  | 0.00          | 0.99 |

heterozygous for the restorer gene. However, in the reciprocal crosses using the same 5 selected  $BC_3F_2$  plants as females x 'Sprite' pollen (frfr), 100% of the  $F_1$  progeny were fully fertile (10 progeny per cross for 50 plants total observed). To summarize: (1) CMS-Sprite (frfr) x selected  $BC_3F_2$  (Frfr) segregated 50% semisterile (Frfr) and 50% sterile (frfr). (2) Selected  $BC_3F_2$  (Frfr) x 'Sprite' (frfr) produced 100% fertile  $F_1$  progeny.

The fertile  $F_1$  progeny from the cross  $BC_3F_2$  x 'Sprite' were allowed to set  $F_2$  seed. Assuming 1/2 the  $F_1$  progeny to be heterozygous for the restorer gene, one would expect segregation for frfr (sterile) progeny in the  $F_2$  generation. One hundred  $F_2$  plants from each of three crosses, a total of 300  $F_2$  plants, were observed and all were fully fertile (Table 4). The five selected  $BC_3F_2$  used in reciprocal crossing were also allowed to set  $BC_3F_3$  seed. Three  $BC_3F_3$  populations of 40 plants each, a total of 120 plants, were grown and again no male sterile plants were produced; all  $BC_3F_3$  plants were fertile (Table 4).

These results are consistent with a hypothesis that restoration of CMS is an irreversible phenomenon. Once full restoration is achieved in the  $F_2$  generation, segregation for sterility in subsequent generations is no longer observed. This would suggest that cytoplasmic-nuclear interaction in semisterile heterozygotes produces a "permanent" cytoplasmic alteration leading to full fertility in the next generation.

Table 4. Inheritance of fertility restoration on sterile cytoplasm in testcrosses and derived generations of 5 selected fertile  $BC_3F_2$  plants heterozygous for the restorer gene.

| Population                                  | No. of<br>plants | Classification |
|---------------------------------------------|------------------|----------------|
| 2-24-13-7-1 x Sprite                        | 10               | 100% fertile   |
| 2-24-13-7-12 x Sprite                       | 10               | "              |
| 2-24-13-7-14 x Sprite                       | 10               | "              |
| 2-19-44-20-12 x Sprite                      | 10               | "              |
| 2-19-44-3-3 x Sprite                        | 10               | "              |
| 5 fertile $BC_3F_2$<br>x Sprite ( $P_2$ )   | 300              | 100% fertile   |
| 5 fertile $BC_3F_2$<br>selfed ( $BC_3F_2$ ) | 120              | 100% fertile   |

The question remains as to why segregation in a ratio of 3:1 (fertile:sterile) takes place in the  $F_2$  generation using R-351 restorer. Since the  $F_1$  generation (CMS-Sprite x Fr/-) only results in semisterility, the cytoplasm is not yet fully restored. The condition of semisterility appears to be an intermediate stage, with only partial cytoplasmic function (with respect to microsporogenesis) or perhaps with a heterogeneous population of functional and dysfunctional organelles. Segregation of the nuclear restorer in the  $F_2$  generation may be accompanied by cytoplasmic sorting of functional and dysfunctional organelles. The few  $F_2$  plants (less than 10%) appearing semisterile may be those heterozygous for nuclear restorer (Frfr) yet with cytoplasmic organelles not yet fully sorted or fully restored (Fig. 1).

One assumption made in analyzing restorer gene action in Phaseolus is that organelles are maternally inherited. Although biparental inheritance of chloroplasts in Phaseolus has been suggested (Parker, 1933), restriction pattern analysis of mitochondrial DNA indicates strict maternal inheritance of mitochondria in Phaseolus (see Chapter 3).

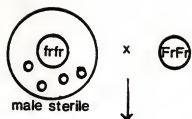
### Conclusions

With the characterization of restoration in CMS-Sprite by R-351, two genetic systems of restoration have been described for the sterile G08063 cytoplasm. The first,

Figure 1. Diagrammatic representation of a hypothesis for cytoplasmic-nuclear interaction resulting in irreversible restoration of fertility to CMS-Sprite by R-351 restorer line.

○ : organelles dysfunctional at microsporogenesis

● : organelles functional



F1: partial restoration



semisterile

self-pollination

F2: segregation to 3:1 (fertile:sterile)

25%

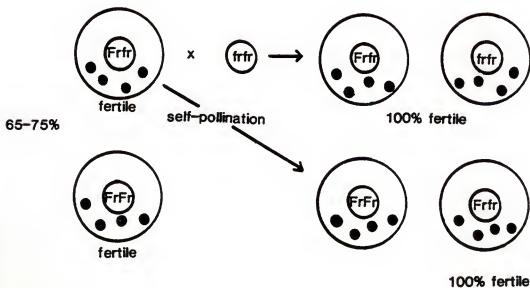


male-sterile

10%



semisterile



65-75%

fertile

self-pollination

100% fertile

fertile

100% fertile

consisting of at least 3 genes showing complementary and additive gene action, has been described previously (Mackenzie, 1984). The second source of restoration appears to be controlled by a single gene. To my knowledge the only other report of restoration by two distinct restorer systems exists in Petunia. In CMS petunia, restoration of fertility can occur using a polygenic restorer (Izhar, 1978), or by a single gene restorer (Edwardson and Warmke, 1967).

In CMS-Sprite, restoration by R-351 appears to be an irreversible cytoplasmic change.  $F_2$  populations segregate to 3:1 (fertile:sterile). Once the cytoplasm has been fully restored ( $3/4$  of  $F_2$ ),  $2/3$  of the fertile  $F_2$  plants are heterozygous for the nuclear restorer gene. Yet, no phenotypic segregation for sterility occurs, either by testcross (fertile  $F_2$  x maintainer) or self-pollination.

Irreversible restoration has been observed in CMS Vicia faba, but full restoration is achieved in the  $F_1$  generation with no subsequent segregation in  $F_2$  progeny (Bond et al., 1966). Thiellement (1977) describes intermediate phenotypes producing sterile and fertile flowers on a single plant, similar to the semisterile class in CMS-Sprite. In Vicia faba Thiellement (1982) suggests the possibility of heterogeneous organelle populations in these intermediate types. However, the CMS phenotype in V. faba is associated with the appearance of virus-like double-stranded RNA

molecules in the cytoplasm. These RNAs disappear upon reversion or nuclear restoration (Grill and Garger, 1981). Although large double-stranded RNAs have been observed in CMS-Sprite (G08063) cytoplasm, no relationship has yet been found between their presence and male sterility (Mackenzie, 1984).

Line R-351 provides a simply-inherited restorer gene system which is convenient to manipulate. The lack of full restoration in an  $F_1$  generation limits the usefulness of this restorer in hybrid seed production. However, if continued investigation of restoration by R-351 supports the hypothesis of irreversible restoration by cytoplasmic alteration, CMS in Phaseolus vulgaris may provide a favorable system for study of direct cytoplasmic-nuclear interaction.

CHAPTER IV  
CHARACTERIZATION OF CYTOPLASMIC DOUBLE-STRANDED  
RNAs IN CMS Phaseolus vulgaris L.

Introduction

Early studies of cytoplasmic male sterility (CMS) provide evidence that viral interaction may be involved, perhaps even causative, in CMS (Atanasoff, 1971). Graft transmissibility of male sterility is reported in CMS petunia (Corbett and Edwardson, 1964), alfalfa (Thompson and Axtell, 1978), sunflower (LeClercq, 1971), and sugar beet (Curtis, 1967). In the case of sugar beet, meristem culture and heat treatment are effective in restoring fertility (Lichter, 1978).

Tests for viral involvement in Vicia faba led to the observation of cytoplasmic "spherical bodies", of about 70 nm in diameter (Edwardson et al., 1976), containing double-stranded RNA (dsRNA) (Scallia et al., 1981; Grill and Garger, 1981). Upon reversion or nuclear restoration to fertility the dsRNAs are no

longer detectable and restoration is no longer dependent upon nuclear genotype (Bond et al., 1966). Selection for a restorer gene is, perhaps, selection for viral resistance.

In some cases, virus-like particles do not appear causative but may interact with expression of male sterility. In CMS maize, line LBN contains dsRNAs that possibly confer a higher degree of sterility (Sisco et al., 1984). In pepper, CMS plants heterozygous for a nuclear restorer gene, normally fertile, appear semi-sterile or sterile after infection by virus (Ohta, 1970).

However, presence of dsRNA in CMS plant material may show no relationship to male sterility. In CMS beet, large mitochondrial dsRNAs have been reported but they occur in both male fertile and male sterile plants (Powling, 1981). Double-stranded RNAs occur in CMS sunflower but also in male fertile plants (Brown et al., 1986). Cryptic, or temperate RNA viruses are defined as RNA viruses producing no detectable disease symptoms, present in low concentration, nearly 100% seed-transmissible, and showing no evidence of graft-transmissibility. They occur in sugar beet, carnation, faba bean, and at least 15 other

species (Boccardo et al., 1983; Lisa et al., 1981; Natsuaki et al., 1983; Dodds et al., 1984).

In CMS Phaseolus vulgaris two large dsRNAs, ca. 15.4 and 16.2 kilobases in size were observed (Mackenzie, 1984). The RNAs were in high concentrations, and purified with the mitochondrial fraction. They were also present in fertile revertant lines. Two dsRNAs of comparable size were reported in the cv. Black Turtle Soup of Phaseolus vulgaris (Wakarchuk and Hamilton, 1985). Whether the dsRNAs in CMS-Sprite are related to those reported by Wakarchuk and Hamilton (1985), and their mechanism of transmission are the questions of this study.

#### Materials and Methods

The large dsRNAs were observed in line G08063, reported to be the fertile accession line from which the CMS source was derived (Singh et al., 1980). The RNAs are also present in CMS-Sprite, produced by recurrent backcrossing of the original CMS line to 'Sprite' snap bean. 'Sprite' snap bean variety did not show evidence of the large RNAs.

Pollen transmissibility. Fertile 'Sprite' plants were pollinated using pollen from line G08063 . Ten  $F_1$  plants were grown and allowed to set  $F_2$  seed in the greenhouse to prevent insect pollinations.

Graft transmissibility. Plants were selected at 14 to 16 days for grafting. Grafts of 10 fertile 'Sprite' scions to CMS-Sprite root stocks (Sprite/CMS-Sprite) and 2 CMS-Sprite scions to 'Sprite' root stocks (CMS-Sprite/Sprite) were made using a razor blade to form a v-shaped cleft graft. The graft point was supported by wrapping in plastic wrap and masking tape. Grafts were then staked and misted 3 to 4 times daily for 2 weeks. The plants were also covered with small plastic bags to maintain high humidity for the first 7 to 10 days after grafting. Grafts were grown under fluorescent and incandescent lights (16 hour daylength) at room temperature.

When the graft unions set, the plants were transferred to the greenhouse and allowed to set selfed seed ( $S_1$ ). The  $S_1$  generation was grown in the greenhouse and allowed to set selfed ( $S_2$ ) seed.  $S_3$  progeny from two Sprite/CMS-Sprite grafts were tested for the presence of the dsRNAs.

Isolation of dsRNAs. The dsRNAs were isolated in highest concentration using differential centrifugation

and retaining the mitochondrial fraction. Isolation procedures were as described by McNay et al. (1983). Deoxyribonuclease was used for digestion of extramitochondrial DNA. Seeds were germinated and grown in the dark for 1 week. Typically 100 g hypocotyl tissue was homogenized in a Waring blender at maximum speed using 5 1-second grindings. Phenol extraction of nucleic acids was followed by ethanol precipitation prior to gel electrophoresis.

Agarose gel electrophoresis. Nucleic acids from 3-10 g tissue were electrophoresed in horizontal submarine agarose gels, 20 x 25 cm, in TPE (0.036 M Tris, 0.030 M  $\text{NaH}_2\text{PO}_4$ , 0.001 M  $\text{Na}_2\text{EDTA}$ ). Agarose gels (0.8%) were run at 2.0 V/cm for 16 hours.

Gels were stained in an ethidium bromide solution (500 ug/ml) for 60 minutes and photographed over 302 nm ultraviolet light with Polaroid 55 film and Wratten 23a and 9 filters.

### Results

Since CMS-Sprite is maintained by recurrent backcrossing to 'Sprite' snap bean, a cultivar that does not carry the large dsRNAs, seed transmissibility of the RNAs to progeny can be assumed. In crosses between 'Sprite' and G08063 (pollen donor), pooled  $\text{F}_2$  progeny

from 10  $F_1$  plants showed a high concentration of the large dsRNAs indicating pollen transmissibility (Fig.2).

Using the same isolation and electrophoresis procedure with 'Black Turtle Soup', two large dsRNAs were observed, presumably the same as those described by Wakarchuk and Hamilton (1985). These comigrated with the dsRNAs in line G08063 (Fig. 2). Although the RNAs can be isolated in highest concentration using a mitochondrial DNA isolation procedure, they do not appear to be contained within the mitochondrion (Fig. 2, D)

Introduction of a fertility restorer gene has no apparent effect on the presence of the dsRNAs in CMS-Sprite. Tests of  $F_3$  populations produced by crossing CMS-Sprite with restorer line R-351 (see chapter 1) indicated that fully fertile restored progeny still contained the dsRNAs (data not shown).

Six of the Sprite/CMS-Sprite and two CMS-Sprite/Sprite grafts survived. Grafting experiments indicated that neither CMS nor dsRNAs were graft-transmissible. Sixty  $S_3$  plants (10 per graft) from Sprite/CMS-Sprite were grown and all were fully fertile.  $S_3$  progeny from two grafts (Sprite/CMS-Sprite) were tested and showed no evidence of the RNAs (Fig. 2).

Figure 2. Agarose gel electrophoresis of A,B)  $S_3$  generations from two grafts (Sprite/CMS-Sprite), C) fertile  $G_0$ 8063, D)'Black Turtle Soup', and E)  $F_2$  generation from 'Sprite' x  $G_0$ 8063. (Smaller RNA molecules present in 'Sprite' are also visible in lane E.)



### Discussion

The dsRNAs were seed and pollen transmissible. The RNAs did not appear to be unique to line G08063; they were similar to those reported by Wakarchuk and Hamilton (1985) in 'Black Turtle Soup'. It is possible that, at some point in the past, 'Black Turtle Soup' and line G08063, a white-seeded type, were grown in proximity for transfer of the RNAs or derived from a population containing the RNAs.

Presence of the RNAs in fertile G08063 and the CMS material is consistent with considering G08063 as fertile progenitor of the CMS source. Their presence in fertile restored and in spontaneous revertant lines (Mackenzie, 1984) shows no direct relationship between the dsRNAs and male sterility. There was no evidence of graft transmissibility of the RNAs.

These RNAs may be another example of a cryptic virus. The RNAs were present in much higher concentration and of greater size, however, than cryptic viruses reported in other species (Francki et al., 1984). Whether they have any effect on plant vigor has yet to be tested.

CHAPTER V  
INSTABILITY AND MITOCHONDRIAL DNA  
REARRANGEMENT IN CYTOPLASMIC MALE STERILE,  
REVERTANT, AND RESTORED Phaseolus vulgaris L.

Introduction

Cytoplasmic male sterility has been associated with mitochondrial alteration in a number of species (for reviews see Leaver and Gray, 1982; Hanson and Conde, 1985). Restriction endonuclease analysis has been useful in these experiments. Mitochondrial DNA restriction patterns may differ substantially, whereas chloroplast DNA restriction patterns may show few detectable differences between fertile and CMS lines (Levings and Pring, 1976; Pring and Levings, 1978; Conde et al., 1982). It is, however, important to note the role nuclear background may play in mitochondrial restriction polymorphism. A prerequisite to analysis of mitochondrial rearrangements relating to CMS is that both fertile and sterile lines share the same nuclear genotype.

An example of nuclear effects on mitochondrial genome activity is found in spontaneous reversion frequencies. Spontaneous reversion to fertility occurs in CMS plants of a number of species (Laughnan and Gabay, 1973; Burton, 1977; Bond et al., 1966; LeClerq, 1971; 1979). The rate of reversion can change significantly as the sterile cytoplasm is backcrossed to different nuclear backgrounds (Clement, 1975; Laughnan et al., 1981).

The most useful fertile materials for investigating the molecular lesions responsible for CMS generally result from spontaneous reversion events. Many spontaneous reversion events are cytoplasmic; this results in a plant which is theoretically identical to the CMS line except at the point of mutation for fertility. Mitochondrial DNA restriction patterns from CMS and revertant lines can be compared to identify possible regions of rearrangement.

In nuclear restoration most species show no evidence of cytoplasmic alteration. Rather, nuclear segregation of the restorer results in fertile and sterile progeny. CMS Vicia faba is an exception. CMS Vicia faba is restored irreversibly such that no further segregation for sterility is observed in  $F_2$  and subsequent generations (Bond et al., 1966). This observation suggests that introduction of a nuclear gene may result in cytoplasmic alteration. As CMS in Vicia faba has been correlated with the presence of

cytoplasmic virus-like elements (Edwardson et al., 1976), restoration may simply result in their elimination. However, mitochondrial DNA restriction analysis in CMS Vicia faba reveals differences between sterile and fertile maintainer lines on identical nuclear backgrounds (Boutry and Briquet, 1982).

In CMS Phaseolus vulgaris the accession line from which the CMS source was derived is G08063 (Singh et al., 1980). The CMS source has now been backcrossed at least 6 generations to 'Sprite' nuclear background (CMS-Sprite). Spontaneous reversion has been observed in CMS-Sprite, providing useful material for mitochondrial analysis.

#### Materials and Methods

Materials used in this study include fertile accession line G08063, 'Sprite' snap bean, CMS-Sprite, cytoplasmic revertant lines 83-1 and WPR-1, and fertile restored  $BC_3F_3$  populations derived from CMS-Sprite x R-351 restorer line.

Reversion frequencies. Frequency of reversion was estimated from a planting of 100 seeds of CMS-Sprite-4 (derived by 4 backcross generations of male-sterile G08063 to 'Sprite' snap bean) and 100 seeds of CMS-Triumph-4 (derived by 4 backcross generations of CMS-Sprite-4 to 'Triumph' snap bean) in the field in single-row plots.

Plots of 50 seeds each, at 10 cm spacing, were alternated and maintained under netting to prevent insect pollination.

Mitochondrial DNA isolation. Mitochondrial isolation procedures were generally as described by McNay et al. (1983), utilizing differential centrifugation and deoxyribonuclease digestion of extramitochondrial DNA. Seven-day old dark-grown hypocotyls with cotyledons were used. Approximately 100 g tissue was homogenized in a Waring blender at maximum speed with 5 1-second grindings. A mitochondrial pellet was isolated after 2 10-minute centrifugations at 1000 g followed by a 10 minute centrifugation at 15,900 g.

The isolation procedure diverged at the point of mitochondrial lysis. Mitochondria were lysed in 5 ml of lysis buffer (100 mM Tris, pH 8, 50 mM EDTA, 100 mM NaCl, 1% SDS) with 100 ug/ml proteinase K for 30 minutes at 65°C. Following lysis the DNA isolation procedure is similar to that described by Dellaporta et al. (1983). Chilled 5 M potassium acetate (1/3 volume) was added to the lysis mixture; the mixture was shaken vigorously and chilled on ice 60 minutes, followed by centrifugation at 20,000 g for 20 minutes. The supernatant was filtered through Miracloth (Calbiochem) to a clean tube with 1/2 volume isopropanol and 1/20 volume 5 M ammonium acetate. The DNA was precipitated overnight at -20°C.

DNA was pelleted at 20,000 g for 20 minutes, air-dried,

redissolved in 700  $\mu$ l 50 mM Tris, 10 mM EDTA (pH 8), and transferred to a 1.5 ml microcentrifuge tube. Fifty  $\mu$ l of 3 M sodium acetate and 100  $\mu$ l of 1% cetyl trimethylammonium bromide (CTAB) were added and the tube was centrifuged for 1 minute in a microfuge. The pellet was washed in 70% ethanol and redissolved in 400  $\mu$ l of 50 mM Tris, 10 mM EDTA (pH 8); 50  $\mu$ l of 3 M sodium acetate and 1 ml of absolute ethanol were then added. The DNA was pelleted by centrifugation for 10 minutes in a microfuge, redissolved in 50 mM Tris, 10 mM EDTA (pH 8) and reprecipitated with sodium acetate and absolute ethanol.

Mitochondrial RNA isolation. Cotyledons were removed from 100 g 7-day-old dark-grown hypocotyls. The etiolated hypocotyls were homogenized in a chilled mortar and pestle in homogenization buffer (0.5 M sucrose, 0.005 M  $\text{Na}_2\text{EDTA}$ , 0.1% bovine serum albumin, 0.005 M mercaptoethanol, and 0.05 M Tris-HCl pH 7.5). The preparation was filtered through 4 layers of cheesecloth and two layers Miracloth (Calbiochem) and centrifuged twice at 1000 g for 10 minutes ( $4^\circ\text{C}$ ). The supernatant was centrifuged at 15,900 g 10 minutes to pellet mitochondria. The mitochondrial pellet was resuspended in homogenization buffer and underlayered with two volumes of shelf buffer (0.6 M sucrose, 0.02 M  $\text{Na}_2\text{EDTA}$ , 0.01 M Tris-HCl, pH 7.5). The preparation was centrifuged for 20 minutes at 12,000 g. The pellet was resuspended in shelf buffer and centrifuged at 15,900 g for 10 minutes.

The washed pellet was then resuspended in 6 M guanidium thiocyanate, 0.05 M Tris, 0.01 M EDTA, 2% Sarkosyl, 0.01% 2-mercaptoethanol (Maniatis et al., 1982) and nucleic acids were extracted with phenol and chloroform. The aqueous phase was separated by centrifugation at 12,000 g for 5 minutes. The aqueous phase was then brought to 2.5 ml with 6 M guanidium thiocyanate and mixed with 1 g CsCl (for 2.37 M) (Glisin et al., 1974).

The CsCl mixture was layered over an equal volume of 5.7 M CsCl, 0.1 M EDTA and centrifuged in a SW 50.1 rotor (Beckman) at 32,000 rpm for 12 hours (22°C). Total mitochondrial RNA pelleted and the supernatant was discarded. After air drying, the RNA pellet was resuspended and ethanol precipitated.

#### Agarose gel electrophoresis and hybridizations.

Mitochondrial DNA from approximately 15 g of tissue was electrophoresed in horizontal submarine agarose gels as described in Chapter 2. Bacteriophage lambda dsDNA markers, digested with restriction endonucleases EcoRI, HindIII, and BamHI were included. Digestion procedures were those recommended by suppliers.

DNA was immobilized on nitrocellulose filters as described by Southern (1975). Filters were pre-hybridized at 65°C with 4X SSC, 0.1% SDS, and 50 ug/ml yeast RNA. Total mitochondrial RNA (1 ug) was radiolabelled by

hydrolyzing in glycine hydrolysis buffer for 10 minutes at 90°C. The RNA was 5' end-labelled with [ $\alpha$ -<sup>32</sup>P]ATP (NEN, 7000 Ci/mmol, 10 mCi/ml) using T4 polynucleotide kinase (Bethesda Research Laboratory) for 30 minutes at 37°C (Bedbrook et al., 1977). Unincorporated label was removed from DNA by chromatography through sephadex G-50. Filters were hybridized at 65°C for 16 hours.

Filters were twice washed in 2X SSC and 0.1% SDS. Filters were then twice washed in 0.25X SSC and 0.1% SDS, air-dried, and autoradiographed with intensifier screens at -80°C.

### Results and Discussion

Spontaneous reversion frequencies differed substantially between CMS on 'Sprite' nuclear background (CMS-Sprite-4) and CMS on 'Triumph' nuclear background (CMS-Triumph-4) (Table 5). This difference in reversion rate suggests nuclear control over reversion frequency as described in other CMS systems (Clement, 1975; Laughnan et al., 1981). Three independent fertile revertants from 3 individual CMS-Sprite plants have been selected, testcrossed to CMS-Sprite, and identified as cytoplasmic events; all F<sub>1</sub> progeny (30 plants each) were male-sterile. These revertants have been designated 83-1, WPR-1, and WPR-2.

Table 5. Spontaneous reversion frequencies of CMS-Sprite-4 versus CMS-Triumph-4.

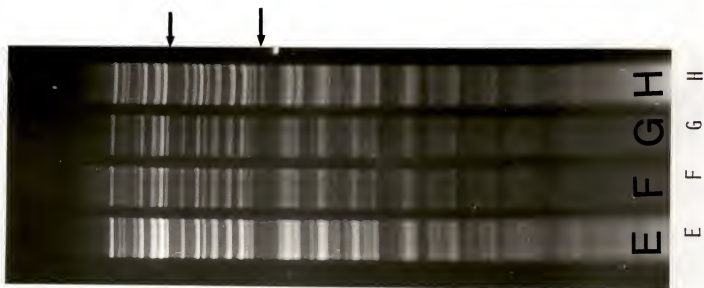
|                                                   | <u>CMS-Sprite-4</u> | <u>CMS-Triumph-4</u> |
|---------------------------------------------------|---------------------|----------------------|
| Number of plants                                  | 60                  | 50                   |
| % plants with at least<br>1 seed pod              | 16.4%               | 50.0%                |
| % reversion events<br>producing >1 seed pod/plant | 20.0%               | 76.0%                |
| Average no. of revertant<br>pods/plant            | 1.2                 | 3.9                  |
| Average no. of seeds/<br>revertant pod            | 3.3                 | 2.3                  |

Frequency of reversion is difficult to measure. For each CMS plant that produces a single seed pod with 4 seeds, only 1 or 2 seeds will develop into a fertile revertant plant. The remainder of the seeds will develop into CMS plants.

Restriction endonuclease analysis of mitochondrial DNA from fertile G08063, CMS-Sprite, and fertile 'Sprite' snap bean revealed differences in restriction patterns between CMS-Sprite and 'Sprite' using a number of restriction enzymes including HindIII, BamHI, SstII, and EcoRI (Fig. 3). No differences were observed between fertile line G08063 and CMS-Sprite. In addition to providing evidence of maternal inheritance of mitochondria in Phaseolus, these findings are also consistent with the report that fertile line G08063 is the progenitor to this source of CMS (Singh et al., 1980).

Two independent CMS-Sprite reversion events, determined to be cytoplasmic, were designated 83-1 and WPR-1. PstI/SstII double digestions of fertile G08063 and CMS-Sprite mitochondrial DNA revealed no differences in restriction pattern. Similar double digestions of revertant WPR-1 and 83-1 mitochondrial DNA revealed the absence of a

Figure 3. Fertile line G08063 (A, E), CMS-Sprite (C, G), fertile revertant 83-1 (B, F) and 'Sprite' (D, H) mtDNA restriction patterns using enzymes HindIII (A, B, C, D) and EcoRI (E, F, G, H). Arrows mark positions of variation.



fragment, approximately 6.5 kilobases in size, that was present in G08063 and CMS-Sprite restrictions (Fig.4). The fact that a similar pattern difference was observed using two different revertants suggests a similar mitochondrial DNA rearrangement may have taken place in both reversion events.

Restriction endonuclease analysis using SstII or PstI enzymes alone revealed no difference in restriction patterns between CMS-Sprite and revertants. However, when mitochondrial DNAs from G08063, CMS-Sprite, and 83-1 were restricted with SstII, blotted to nitrocellulose, and probed with end-labelled total mitochondrial RNA from revertant line 83-1, a high molecular weight band visible in G08063 and CMS-Sprite was not observed in the revertant. This same high molecular weight band was also absent in the revertant when CMS-Sprite RNA was used as probe (Fig. 5), suggesting a DNA rearrangement had occurred as opposed to differential transcription.

Restriction endonuclease analysis of one population of fertile restored  $BC_3F_3$  progeny derived from CMS-Sprite x R-351 restorer line also revealed a mitochondrial DNA rearrangement similar to that revealed in cytoplasmic revertants by SstII/PstI double digestions (Fig.6). Mitochondrial digests of fertile restored plants lacked the 6.5 kb fragment which was present in CMS-Sprite but missing in revertants 83-1 and WPR-1. An unusual feature of the

Figure 4. Mitochondrial DNA restrictions of fertile line G08063 (A, D), CMS-Sprite (B), and fertile revertant lines 83-1 (C) and WPR-1 (E) double-digested with SstII/PstI. Arrows mark position of 5.6 kb fragment.



A B C



D E

Figure 5. SstII restriction endonuclease digestions of mitochondrial DNA from G08063 (A, D), CMS-Sprite (B, E), and revertant 83-1 (C, F) blotted and probed with CMS-Sprite 5' end-labelled total mitochondrial RNA. Arrow marks position of high molecular weight fragment.



A

B

C



D

E

F

Figure 6. Mitochondrial DNA restriction patterns from CMS-Sprite (A) and fertile restored  $BC_3F_3$  progeny (CMS-Sprite x R-351 restorer) (B), using SstII/PstI double digestion. Lambda DNA restricted with BamHI and EcoRI + HindIII (mixed post-digestion) were included as size markers. Arrow marks position of 5.6 kb fragment. (Double-stranded RNA molecules of 15.4 and 16.2 kb are visible in lane A).



A

B

observed rearrangements was that absence of the 6.5 kb fragment in SstII/PstI digests, and of the higher molecular weight fragment in SstII digests, was not accompanied by the appearance of a novel fragment elsewhere in the restriction pattern. There is not yet a clear explanation for this observation.

### Conclusions

The frequency of spontaneous reversion to fertility was altered by a different nuclear background, demonstrating the degree of interaction between nucleus and cytoplasm in expression of CMS. Upon cytoplasmic reversion, mitochondrial DNA rearrangement is reflected in the disappearance of a 6.5 kilobase (kb) PstI/SstII fragment and a high molecular weight SstII fragment. Mitochondrial DNA rearrangements have been observed in revertants of maize CMS-T (Gengenbach et al., 1981; Umbeck and Gengenbach, 1983) and CMS-S (Levings et al., 1980). To my knowledge mitochondrial rearrangements have not been reported in CMS lines restored to fertility. This suggests that in Phaseolus vulgaris introduction of a nuclear gene for restoration causes a cytoplasmic alteration similar to that occurring in spontaneous reversion.

A number of questions are raised by these findings. In restoration the mitochondrial rearrangement may occur by the  $F_2$  generation since the  $F_1$  generation of CMS-Sprite x R-351 restorer was semisterile (see chapter 1). This raises

the question of which form of the mitochondrial genome one would find in the  $F_1$  generation. Also, since no mitochondrial DNA restriction differences were observed between fertile G08063 and CMS-Sprite, perhaps restoration and spontaneous reversion result in a novel mitochondrial genome arrangement. The stability of this novel arrangement might be of interest since some indication of semisterility has been observed in revertant material under high temperatures (data not shown).

Most mitochondrial rearrangements are believed to arise by intra- or inter-molecular recombination events (Lonsdale, 1984). Mitochondrial genomes of the plant species studied exist as circular molecules which are able to recombine into larger multimeric forms or smaller subgenomic molecules (Palmer and Shields, 1984). Such recombination events may explain the high levels of reversion observed on some nuclear backgrounds (Schardl et al., 1985).

## CHAPTER VI CONCLUSIONS

The initial interest in a source of cytoplasmic male sterility (CMS) in Phaseolus vulgaris was for two reasons: the potential use of the CMS line for hybrid seed production, and as a system for study of cytoplasmic-nuclear interaction. Although a simply-inherited source of restoration has now been identified (R-351), full restoration of fertility was not achieved in the  $F_1$  generation of CMS-Sprite  $\times$  R-351 restorer. This makes this CMS system less attractive for hybrid seed development.

In addition to incomplete restoration in the  $F_1$  generation by this restorer gene, restoration by R-351 also appears to be irreversible. Once full restoration was achieved in the  $F_2$  generation, no further segregation for sterility was observed, either in testcrosses or selfed populations. One can then hypothesize that restoration is due to a cytoplasmic alteration brought about by introduction of a nuclear restorer gene.

Cytoplasmic reversion to fertility was also observed in this CMS source. Mitochondrial DNA

rearrangements were observed in these mutants that were identical to those observed upon full restoration.

Although two large cytoplasmic double-stranded RNA molecules were observed in the CMS material, they also occur in related fertile material. The RNAs (approximately 15 and 16 kilobases) were pollen transmissible, were not graft transmissible, and were found in relatively high concentration in the leaf tissue.

Cytoplasmic male sterility in Phaseolus vulgaris does not appear to be due to, nor influenced by, virus infection. CMS is probably the result of a mitochondrial mutation. The novel mitochondrial DNA (mtDNA) restriction patterns observed in spontaneous revertant and nuclear restored plants may be due to processes involved in regaining pollen fertility. At this point some questions arise that can be approached. For example, if the novel mtDNA restriction patterns observed in cytoplasmic revertants and restored plants occur in the process of regaining fertility, the 6.5 kilobase PstI/SstII fragment that disappears in reversion may provide a region of the mitochondrial genome on which to focus. This region can be characterized for genome structure and transcriptional alterations.

A unique aspect of the results obtained is the suggestion that the nuclear restorer interacts with the cytoplasm to bring about cytoplasmic rearrangement. An approach to understanding how this may come about is in analysis of semisterile  $F_1$  progeny (CMS-Sprite x R-351 restorer). Semisterility may indicate an intermediate state in the process of restoration of fertility.

CMS in Phaseolus vulgaris provides a useful system for analysis of cytoplasmic genome expression in higher plants. Although similarities exist between CMS mutants in different species, a number of differences are known. It is likely that CMS is a common phenotype for a number of very different mutational events. In other words, there exist a number of interactions taking place between the nucleus and organelles. When interrupted, a minor consequence may be loss of microsporogenesis; perhaps an alternative consequence is lethality. Determining how these interactions take place may lead to a more complete understanding of the evolution of organelles.

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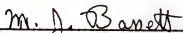
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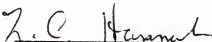
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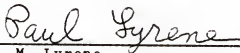
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